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**BRCA1 Is a Histone-H2A-Specific Ubiquitin Ligase**

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**SUMMARY**

The RING domain proteins BRCA1 and BARD1 comprise a heterodimeric ubiquitin (E3) ligase that is required for the accumulation of ubiquitin conjugates at sites of DNA damage and for silencing at DNA satellite repeat regions. Despite its links to chromatin, the substrate and underlying function of the BRCA1/BARD1 ubiquitin ligase remain unclear. Here, we show that BRCA1/BARD1 specifically ubiquitylates histone H2A in its C-terminal tail on lysines 127 and 129 in vitro and in vivo. The specificity for K127-129 is acquired only when H2A is within a nucleosomal context. Moreover, site-specific targeting of the BRCA1/BARD1 RING domains to chromatin is sufficient for H2Aub foci formation in vivo. Our data establish BRCA1/BARD1 as a histone-H2A-specific E3 ligase, helping to explain its localization and activities on chromatin in cells.

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**INTRODUCTION**

Breast cancer-associated protein 1 (BRCA1) is a key mediator in the DNA damage response, which is linked to a wide range of functions that serve to maintain genomic stability. Cellular BRCA1 forms a heterodimer with BRCA1-associated RING domain 1 (BARD1) (Wu et al., 1996), which promotes the repair of double-stranded DNA breaks through homologous recombination (Moynahan and Jasin, 2010) and contributes to the DNA-damage-induced G2/M checkpoint (Ku et al., 2001). Loss of BRCA1 function in cells results in hypersensitivity to DNA damage and accumulation of chromosomal aberrations associated with the development of cancer (Venkitaraman, 2001).

In vitro studies have shown that the RING domains of BRCA1/BARD1 possess a ubiquitin ligase (E3) function (Ruffner et al., 2001), but a bona fide substrate for this activity is still lacking. During replication and after treatment with agents that damage DNA, BRCA1 and BARD1 colocalize in discreet nuclear foci with ubiquitin conjugates (Morris and Solomon, 2004). However, it is not known whether these conjugates are a product of BRCA1/BARD1 E3 activity or arise through the function of several other E3 proteins that also colocalize at sites of DNA double-strand breaks as part of a ubiquitin-mediated DNA-damage-signaling pathway (Doil et al., 2009; Mattirolli et al., 2012; Stewart et al., 2009).

Recently, Zhu et al. (2011) reported that defects in BRCA1 E3 function are linked with a derepression of satellite DNA that is accompanied by decompaction of chromatin and reduced levels of ubiquitylated histone H2A (H2Aub). Moreover, these phenotypes can be reversed by exogenous expression of histone H2A protein fused to ubiquitin. However, a direct role for BRCA1/BARD1-dependent ubiquitylation of histones was not established.

We investigated the interaction of BRCA1/BARD1 with chromatin and found that it involves a highly specific histone H2A-ubiquitin ligase that modifies previously uncharacterized lysines in the C-terminal tail of H2A. We discuss these observations in light of the known biological functions of BRCA1.

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**RESULTS**

**BRCA1/BARD1 Ubiquitylates H2A in Nucleosomes**

Although BRCA1 binds to DNA in a sequence-independent manner in vitro (Paull et al., 2001), in cells, it is most commonly found at DNA breaks associated with chromatin. Accordingly, we found that purified recombinant BRCA1/BARD1 bound nucleosome substrates in an electrophoretic mobility shift assay (EMSA) (Figures S1A–S1F). The binding was dynamic, as compared with the activity of the RING1B/MEL18 subunits of PRC1 (Figures S1A and S1B). This binding, along with the known structural similarities between the RING domains of BRCA1/BARD1 and those of the histone-H2A-specific ubiquitin ligase complex Polycomb repressive complex 1 (PRC1) (Buchwald et al., 2006; Figure S1G), prompted us to investigate whether BRCA1/BARD1 ubiquitylates nucleosomal histone proteins.

We examined BRCA1/BARD1 E3 activity on individual histone proteins and reconstructed nucleosome substrates in vitro and compared it with the activity of the RING1B/MEL-18 subunits of PRC1 (Figures 1A and 1B). As reported previously, RING1B/MEL18 monoubiquitylated individual histone proteins with similar efficiency in vitro, but ubiquitylated nucleosome substrates specifically on K118-119 of H2A (Figure 1A). This reflects the known cellular specificity of PRC1 for K118-119 of H2A.
RING (residues 26–126)-Gly2SerGly2-BRCA1 RING (residues 100–200) complex (Christensen et al., 2007), to ubiquitylate histone H2A. To establish that BRCA1/BARD1 can ubiquitylate histone H2A predominantly at lysine 119 in its C-terminal tail both in vitro and in vivo (Elderkin et al., 2007).

BRCA1/BARD1 ubiquitylates human histone H2A at a novel site in vitro and in vivo. Next, we wished to identify the lysine residue on histone H2A that is modified by BRCA1/BARD1. Recently, RNF168 was shown to monoubiquitylate the N-terminal tail of histone H2A on lysines K118-119 (Mattiroli et al., 2012). However, this was not the case for BRCA1/BARD1, which efficiently ubiquitylated nucleosomes reconstituted with H2A deleted for its N-terminal tail (Figures S3B and S3C). In contrast, RING1B/MEL18 ubiquitylates histone H2A predominantly at lysine 119 in its C-terminal tail both in vitro and in vivo (Elderkin et al., 2007).

We tested recombinant Xenopus laevis nucleosomes containing H2A mutated in different lysines at its C-terminal tail. Whereas RING1B/MEL18 did not ubiquitylate nucleosomes reconstituted with mutant H2A K118-119R protein in vitro (Figure 3A; Elderkin et al., 2007), these same mutant nucleosomes were efficiently ubiquitylated by BRCA1/BARD1 (Figure 3B), indicating that it could modify residues other than K118 and K119. However, BRCA1-dependent ubiquitylation was markedly reduced with nucleosomes in which both K124 and K127 of H2A were mutated to arginine (H2A K124-127R) (Figure 3B). Ubiquitylation was further reduced in an H2A K124-127-129R mutant, suggesting that BRCA1/BARD1 ubiquitylates histone H2A in vitro at one or more of three residues (K124, K127, and K129). A direct comparison of H2Aub generated by RING1B/MEL18 and that produced by BRCA1/BARD1 in vitro revealed a small difference in migration after SDS-PAGE, providing further evidence that H2Aub produced by BRCA1/BARD1 in vitro occurred at a different lysine residue (Figure 3C).

To determine which of the lysines in the H2A C-terminal tail is the preferred site of ubiquitin conjugation by BRCA1/BARD1, we ubiquitylated chromatin purified from chicken erythrocytes in vitro and analyzed the products by mass spectrometry. We were able to identify ubiquitylation of the C-terminal peptide KAK (residues 126–128 of chicken H2A; Figures 3G, top, and S3E). Coupled with the detection of unmodified lysine 128, this suggested that K126 of chicken H2A (equivalent to K127 in Xenopus and human) might be the predominant residue for BRCA1/BARD1-dependent ubiquitin conjugation in vitro.

BRCA1/BARD1 ubiquitylates human histone H2A at K127 and K129 in vivo. The most abundant form of H2A ubiquitylation in cells is K118-119. To determine the specific lysine in histone H2A that is ubiquitylated by BRCA1/BARD1 in vivo, we used DT40 cells stably expressing wild-type or mutant forms of FLAG-H2A. FLAG-H2A protein was immunoprecipitated with anti-FLAG, and western blots were probed with antibodies against H2Aub (E6C5),
ubiquitin (FK2), or FLAG epitope (Figure 3D). This confirmed that mutant FLAG-H2A K118-119R was ubiquitylated, albeit at a greatly diminished level compared with wild-type H2A. Ubiquitylation was further reduced in cells expressing FLAG-H2A K118-119-125-127-129R, confirming ubiquitylation of H2A at its C-terminal tail in vivo (Figure 3D).

We next expressed the separate BRCA1 and BARD1 RING domains (BC-R/BD-R) in DT40 cells with different mutant FLAG-H2A proteins. In untransfected cells expressing wild-type FLAG-H2A, western blots revealed a single band corresponding to H2AubK119 (band 1; Figure 3E). Upon transfection with BC-R/BD-R, we observed a second, slower-migrating band (band 2) similar to that observed after ubiquitylation of H2A by BRCA1/BARD1 in vitro. Whereas band 1 was absent in cells expressing mutant FLAG-H2A K118-119R, band 2 was induced upon expression of BC-R/BD-R in these cells. Band 2 was not observed in cells expressing FLAG-H2A K124-127-129R mutant, suggesting that ubiquitylation required one or more of the three most C-terminal lysine residues of H2A. We observed a similar requirement for K125, K127, and K129 for ubiquitylation of FLAG-H2A K118-119R in HEK293 cells expressing the BDfBC RING domain complex (Figure S3D). Although these data highlight the importance of K127 for ubiquitylation of H2A, they did not establish which of the lysine residues (K125, K127, and K129) becomes conjugated to ubiquitin.

Given that H2Aub comprises only 5%–10% of all H2A in cells, the amount of H2Aub that was modified at lysine residues other than K118-119 was extremely low (Figures 3D and 3F). Expression of the RING fusion protein BDfBC in HEK293 cells significantly increased the cellular pool of H2Aub, indicating that H2A is an efficient substrate for this E3 even when it is not specifically directed to chromatin as a fusion protein with LacI (Figure 3F). By contrast, we observed no increase in H2Bub after expression of BDfBC (Figure S2B). Next, we purified chromatin-associated H2Aub from cells expressing BDfBC and analyzed it by mass spectrometry. The number and close proximity of lysine

![Figure 2. Ubiquitylation of Histone H2A by BRCA1/BARD1 E3 Activity In Vivo](image-url)
residues required us to digest H2Aub with pepsin at pH 1.3 rather than trypsin prior to mass spectrometry analysis. We found that although the majority of cellular H2Aub was modified on K118 or K119, expression of BDfBC coincided with the recovery of ubiquitylated peptide corresponding to the C-terminal residues GK (residues 128-129) of H2A (Figures 3G, bottom, and S3F). We also identified peptides consistent with low-level ubiquitin conjugation at K127 (Figure S3G). On the basis of these data, the primary acceptor for BRCA1 E3 activity is probably lysine 129 of histone H2A. However, lysine 127, which is important for efficient ubiquitylation of H2A by BRCA1/BARD1 in vitro and in vivo, may also be ubiquitylated.

**DISCUSSION**

To date, neither the substrate nor the function of the BRCA1/BARD1 ubiquitin ligase has been well established. We have established that BRCA1/BARD1 specifically ubiquitylates histone H2A in chromatin in vitro and in vivo. Our data support a role for BRCA1/BARD1 as a histone-H2A-specific ubiquitin ligase.
that ubiquitylates the C-terminal tail of H2A at the previously uncharacterized lysine residues K127-129.

Several pieces of evidence link BRCA1-dependent E3 activity with ubiquitylation of histones in chromatin. First, purified BRCA1/BARD1 ubiquitylates individual histone proteins in vitro, albeit with little specificity (Chen et al., 2002; Hashizume et al., 2001; Mallory et al., 2002). We show that the specificity of BRCA1/BARD1 for K127-129 of H2A is acquired only in a nucleosomal context, indicating that histone-H2A-specific ubiquitin ligase PRC1 (Brzovic et al., 2006; Buchwald et al., 2006; Li et al., 2006). Of note, the basic patches on the surface of RING1B/M1, which have been shown to be involved in DNA binding of the E3-UbcH5 complex on nucleosomes (Bentley et al., 2011), are conserved in BRCA1, but not in BARD1. However, it is unclear whether this might affect the position of E3 relative to its nucleosome substrate.

We note that BRCA1/BARD1 and RING1B/MEL18 are heterodimeric E3 ligases (Brzovic et al., 2001; Buchwald et al., 2006; Li et al., 2006), whereas RNF168 is monomeric (Campbell et al., 2012) and therefore might interact with its chromatin substrate in a different manner (Mattiroli et al., 2012). Recent evidence suggests that ubiquitylation of histone H2A by RING1B/M1 and RNF168 is dependent on an acidic patch present on the exposed surface of nucleosomes. Expression of a peptide that interfered with binding to this acidic patch caused a reduction in DNA damage-induced H2Aub by RNF168 and a concomitant failure to recruit BRCA1 at sites of DNA damage in vivo (Leung et al., 2014; Mattiroli et al., 2014). However, recruitment of BRCA1 at sites of DNA damage is dependent on RNF168-mediated ubiquitylation, and no conclusion can be made regarding the effect on BRCA1 E3 ligase activity.

Third, our data support recent evidence indicating that small interfering RNA (siRNA)-mediated knockdown of BRCA1 results in derepression of satellite DNA with an accompanying loss of H2Aub that might be uniquely associated with the E3 activity of this protein, we established that generation of H2Aub by BDfBC is biochemically active BRCA1.

EXPERIMENTAL PROCEDURES

Cell Culture

DT40 chicken cells were propagated in standard media supplemented with RPMI (Invitrogen) at 37°C, 6% CO2. HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum. HEK293T T-Rex cells (Invitrogen) were grown in DMEM containing 10% tetracycline-free fetal bovine serum.

Microscopy

Cells were prepared as described in the Supplemental Experimental Procedures and visualized using an LSM510 confocal microscope (Leica).

Ubiquitylation of Recombinant Nucleosomes and Chromatin

Recombinant X. laevis histones were expressed and purified from E. coli and reconstituted into nucleosomes as described in the Supplemental Experimental Procedures. The chicken histone octamers were a kind gift from Professor Daniela Rhodes and were purified from chicken erythrocyte nuclei as described previously (Thomas and Butler, 1980). For nucleosome ubiquitylation, chromatin or individual histones were incubated with 200 ng E1 (affinity or Boston Biochem), 200 ng UbC-H5c (affinity or Boston Biochem), 1 μg
ubiquitin (Sigma), 1 mM ATP and 0.1 μg purified E3 were in a reaction volume of 10 μl for 15 min, followed by addition of 10 μl of 0.5 μg 125I-ubiquitin, substrate and 1 mM in 1x ub buffer (Mallery et al., 2002). Reactions were stopped by addition of SDS buffer and applied for gel electrophoresis.

**Purification of Ubiquitylated Histones and Mass Spectrometry.**

H2Aub was isolated from cells by initial purification of H2A and H2B from chromatin in cells using the Histone Purification Kit (Active Motif). Histones were separated by PAGE using 12% Bis-Tris gel (Invitrogen) and MES buffer, and Coomassie-stained H2Aub bands were excised. In-gel digestion with pepsin (pH 1.3) was performed and peptides were analyzed by nanoLC-LTQ/Orbitrap in a data-dependent tandem mass spectrometry mode. For nucleosomes ubiquitylated in vitro, in-gel digestion was also performed with pepsin and analyzed by NextGen Bioscience.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.07.025.

**AUTHOR CONTRIBUTIONS**


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